Antidiabetic potential of *Naregamia alata* Wight & Arn.

Final synopsis of Ph.D Thesis

To be submitted for the award of degree of

Doctor of Philosophy

in

Faculty of Science and Technology

(APPLIED BOTANY)

By

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November 2022

Synopsis for Ph.D Thesis

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Title of the PhD thesis	Antidiabetic potential of *Naregamia alata* Wight & Arn.
Work Place	Dept of Applied Botany Mangalore University Mangalagangothri – 574199
Date of Registration	23/4/2018
PhD Registration No.	MU/EXB/Ph.D./CR.81/A.B./2019-20/E.13
Nature of Registration	Full Time
Subject	Applied Botany

Antidiabetic potential of *Naregamia alata* Wight & Arn.

1. Introduction

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycaemia resulting from defects in insulin secretion, resistance to insulin or both with altered protein, carbohydrate and lipid metabolism (Akram and Hisham, 2015). Diabetes mellitus has drawn much attention of researchers due to its increasing mortality and associated complications (Weiwei et al., 2017). According to the International Diabetes Federation, diabetes mellitus has become a major threat to the health and the third biggest killer after cardiovascular diseases and cancer. It is estimated that the worldwide increase in diabetes mellitus will reach to 366 million in 2030 from 171 million in 2000. In this context more research efforts are required to promote a better management of people with diabetes mellitus (Latha and Vijayakumar, 2019). The medicinal plants and herbal preparations are gaining more attention due to their pharmacological actions in controlling various disorders (Vikram et al., 2014). Since ancient times, medicinal plants have been used in the treatment and management of diabetic mellitus in traditional medicine system throughout the world (Chikezie et al., 2015). The secondary metabolites present in the plants, play a major role in different pharmacological and biological activities (Wink, 2015). Scientific evidences suggest that the plant based phytochemicals provide a basis for the development of novel drugs by trial and error methods with traditional and molecular approaches and, can be used to treat health disorders in human beings (Harvey, 1999; Surendran et al., 2014). Hence, more research needs to be carried out to separate the active phytoconstituents with hypoglycaemic property and analysing their curative properties by using different *in vitro* and *in vivo* models for biological evaluation to support the claim which may help to manage diabetes mellitus (Patel et al., 2012).

Naregamia alata Wight & Arn. (Meliaceae) is one of the most important Ayurvedic herb widely distributed in Western Ghats of India up to an elevation of 1000 m and used in treating wounds, ulcers, vitiated conditions of pitta and vata, rheumatism, itching, halitosis, asthma, bronchitis, splenomegaly, scabies, pruritus, dysentery, dyspepsia, catarrh, anaemia, malarial and chronic fevers (Undarathi et al., 2017). The preliminary study conducted by Hidayathulla (2012), revealed the antidiabetic activity of methanol extract of *N. alata* in streptozotocin induced diabetic rats. Taking the clue from this work, the present work aiming at understanding the hypoglycaemic effect of bioactive compounds in the methanol extract/fractions and their mode of actions in controlling diabetes mellitus was taken up.

Objectives

1. Collection of *Naregamia alata* from the wild, extraction and purification of phytochemicals.
2. Determination of *in vitro* phytopharmacological activity.
3. Evaluation of *in vivo* antidiabetic activity.
4. Mode of action of *N. alata* extract/fraction on diabetes mellitus.
5. Preliminary screening of phytochemicals in the *N. alata* extract/fraction.
6. Characterisation of the antidiabetic compounds in the *N. alata* extract/fraction.
7. Evaluation of *in silico* pharmacokinetics, drug-likeness and medicinal chemistry.

2. Literature Survey

Diabetes mellitus is one of the oldest diseases known to man and it was identified during 400–500 A.D by the ancient Indian physician, Sushruta, and the surgeon Charaka named it Madhumeha, due to sweetness of urine of the diabetic people (Lakhtakia, 2013; Latha and Vijayakumar, 2019). Diabetes mellitus is a group of complex metabolic disorders characterized by chronic hyperglycaemia resulting from defects in insulin secretion, resistance to insulin or both and it alters the metabolism of carbohydrates, lipids, and proteins along with the imbalance of minerals and electrolytes (Kharroubi and Darwish, 2015; Solis-Herrera et al., 2018). Diabetes mellitus is a growing health problem in the world that causes severe morbidity and mortality (Latha and Vijayakumar, 2019). The most accepted classical classification of diabetes mellitus proposed by the American Diabetes Association (ADA) in 1997 are two major types called type 1 and type 2 diabetes mellitus and one minor type called gestational diabetes mellitus (Kharroubi and Darwish, 2015). Among these, type 1 and type 2 diabetes mellitus are most commonly discussed (Salehi et al., 2019). Type 1 diabetes mellitus is called insulin-dependent diabetes or juvenile-onset diabetes, which is caused by an absolute or near absolute deficiency of insulin (Katsarou et al., 2017). The type II diabetes mellitus accounts for 90-95% of those with diabetes, is also called as non insulin dependent diabetes, type 2 diabetes, or adult-onset diabetes, encompasses individuals who have insulin resistance and usually have relative insulin deficiency (International Diabetes Federation, 2013).

Diabetes is known for developing long term complications like cardiovascular diseases, cerebrovascular diseases, renal disorders, inflammation and immunity and obesity. Dyslipidaemia is the serious clinical manifestation associated with diabetes mellitus leads to atherosclerotic cardiovascular disease by increasing serum lipid parameters such as triglycerides, total cholesterol, low-density lipoprotein (LDL)-cholesterol, very low-density lipoprotein (VLDL)-cholesterol and low concentrations of high-density lipoprotein (HDL)-

cholesterol (Hirano, 2018). Diabetic nephropathy is another pathological condition associated with diabetes mellitus which results in renal failure (Lim et al., 2014).

The use of herbal medicine has elicited enormous interest in the educated public and healthcare professionals, even though there is confusion about their identification, effectiveness, therapeutic dosage, toxicity, standardization, and regulation (Sidhu and Sharma, 2013). The World Health Organization (WHO) recommended the use of medicinal plants for the management of diabetes mellitus and encouraged the scientific evaluation of the hypoglycaemic properties of diverse medicinal plants (Chikezie et al., 2015). The ethnobotanical information suggests that about 800 plants may possess anti-diabetic properties (Patel et al., 2012). Plant secondary metabolites like flavonoids, tannins, phenolic, alkaloids, terpenes, steroids and saponins have been reported as antidiabetic agents with different modes of action (Kooti et al., 2016). The major mechanisms in both traditional and western medicines involve a decrease in blood sugar level through stimulating pancreatic β-cells, increasing the affinity and sensitivity of insulin receptors, lowering glycogen breakdown, enhancing glucose utilization within tissues, scavenging the free radicals, resisting lipid peroxidation, correction of the lipid and protein metabolism (Singab et al., 2014; Kooti et al., 2016). Several plants have been studied for their antidiabetic properties by using animal models for example the oral administration of *Aegle marmelos* (L.) Corr. leaf extract to STZ induced diabetic rats showed a potent hypoglycaemic activity by stimulating glucose uptake, enhancing insulin secretion, regeneration of pancreatic beta cells (Maity et al., 2009). The study conducted by Goel et al. (2012), proved that the ethanol extract of *Allium sativum* regulates blood glucose level by normalising the activity of liver hexokinase and glucose-6- phosphatase in addition to normalising liver glycogen, serum insulin, total cholesterol, serum triglycerides, urea and creatinine. Dhanabal et al. (2006), observed the hypoglycaemic effect of the bark extracts of *Pterocarpus marsupium* with reduction in blood glucose level, increase of serum protein and decrease in the levels of cholesterol, triglycerides, alkaline phosphatase, alanine transaminase, aspartate transaminase in alloxan induced diabetic rats. The root of aqueous or alcohol extracts of *Tinospora cordifolia* in alloxan induced diabetic rats produced a significant antidiabetic effect by suppression in plasma glucose, reversal of dyslipidaemia, decrease in serum acid phosphatase, alkaline phosphatase, lactate dehydrogenase (Grover et al., 2002). The study conducted by Akhani et al. (2003) revealed the hypoglycaemic activity of the juice of *Zingiber officinale* rhizome through increasing serum insulin level, suppression of serum cholesterol, triglyceride and blood pressure in STZ induced diabetic rats.

The plant *N. alata* commonly known as Nilanarakam (Malayalam) belongs to the family Meliaceae (Undarathi, 2017; Velayudhan et al., 2015). It is also known as Triparnika and mentioned in Raja Nighantu, moolakadi varga, Flora of Udupi and Indian Medicinal Plants etc. (Mohammed et al., 2017). A small branching undershrub, It is distributed throughout western and southern India (Velayudhan et al., 2015). The Flowers are seen during May and the fruits in October season (Mohammed et al., 2017). This plant has been evaluated for its antimicrobial, hepatoprotective, anti-inflammatory, antioxidant activities (Tripathi et al., 2020). The preliminary work on the antidiabetic activity of methanol extract of this plant in streptozotocin induced diabetic rats was studied by Hidayathulla (2012). However detailed work has not been carried out so far on the mechanism of action of this plant/plant molecules in controlling or the management of diabetes and the phytoconstituets responsible for the antidiabetic activity present in the plant. Hence, the present study deals with the antidiabetic property of *N. alata* and the work is presented in 4 chapters.

3. Chapter 1: Extraction and purification of phytochemicals and *In vitro* pharmacological activity

3.1 Methodology

The whole plant *N. alata* was collected from the natural habitats of Dakshina Kannada district of Karnataka (co-ordinate value: 12.8438 ° N, 75.2479 ° E), India and identified following the Flora of Madras Presidency (Gamble, 1987). A voucher specimen (MU/AB/WJR/01) is kept in the Herbarium of Department of Applied Botany, Mangalore University. The collected plant material was washed, dried and powdered using electrical grinder and stored in an airtight container for further use. The powdered plant sample was extracted in methanol using soxhlet apparatus. The extract was filtered through Whatman number 1 filter paper, dried under vacuum at 45^0C using flash evaporator and stored at 4^0C in airtight containers. Solvent-solvent fractionation of methanol crude extract (MCE) was carried out by modified Kupchan et al. (1973) to obtain petroleum ether (PE), dichloromethane (DCM) and residual methanol (RM) fraction. Further fractionation of DCM was carried out by using column chromatography to obtain fraction 1, 2.1, 2.2.1, 2.2.2 and 3 (Fig 1).

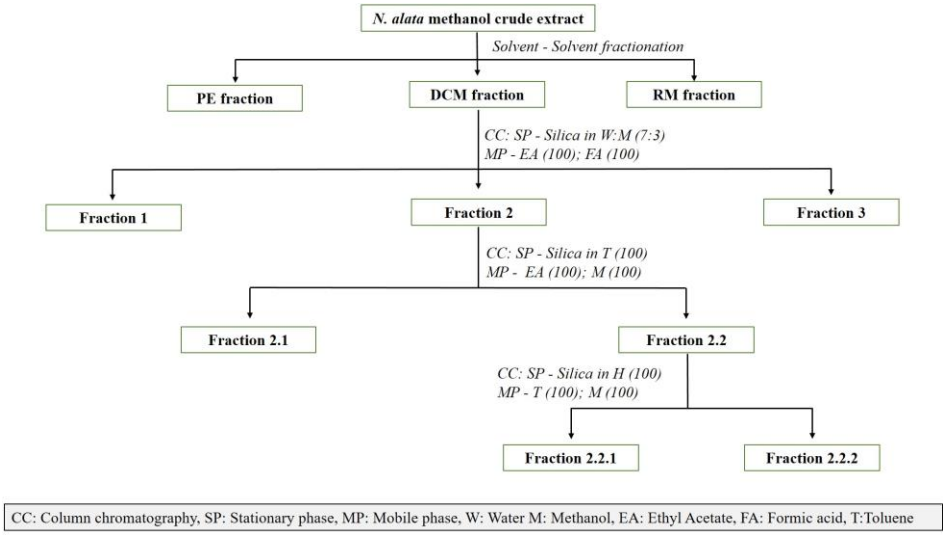
N. alata methanol crude extract
Solvent - Solvent fractionation
PE fraction
DCM fraction
RM fraction
CC: SP - Silica in W:M (7:3)
MP - EA (100); FA (100)
Fraction 1
Fraction 2
Fraction 3
CC: SP - Silica in T (100)
MP - EA (100); M (100)
Fraction 2.1
Fraction 2.2
CC: SP - Silica in H (100)
MP - T (100); M (100)
Fraction 2.2.1
Fraction 2.2.2
CC: Column chromatography, SP: Stationary phase, MP: Mobile phase, W: Water M: Methanol, EA: Ethyl Acetate, FA: Formic acid, T:Toluene

Fig 1. Flow chart showing fractionation of methanolic extract of *N. alata*

The *in vitro* antioxidant activity of the *N. alata* extract/fractions were evaluated using 1,1-diphenyl- 2-picrylhydrazyl (DPPH) free radical scavenging assay (Brand-Williams, 1995). The effect of the *N. alata* extract/fractions on *in vitro* α-glucosidase activity was determined according to the method of Al-Hajj (2016). The modified procedure of McCue and Shetty (2004) was employed to carry out *in vitro* alpha amylase inhibition assay. The *in vitro* uptake of glucose by rat hemidiaphragm was determined by the method described by Hemalatha et al. (2005). Cell viability assay was determined by MTT - (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) based assay (Mosmann, 1987). *In vitro* antibacterial activity of *N. alata* extract/fraction were tested against two gram positive bacteria *Staphylococcus aureus*, *Bacillus subtilis* and three gram negative bacteria *Escherichia coli*, *Pseudomonas aurogenosa* and *Proteus vulgaris*. The *in vitro* anti-inflammatory activity of *N. alata* extract/fractions was determined by using (Human red blood cells) HRBC membrane stabilisation method (Gandhisan et al., 1991).

3.2 Results

In vitro Antioxidant and Antidiabetic activity

In vitro antioxidant assay performed using DPPH radical scavenging method showed the scavenging activity for MCE, DCM, RM, fraction 1, fraction 2.1, fraction 2.2.1, fraction 2.2.2 and fraction 3 with their respective SC_{50} (50% scavenging concentration) values of 508.29 ± 3.87 µg/mL, 402.96 ± 5.10 µg/mL, 425.08 ± 1.84 µg/mL, 339.71 ± 3.14 µg/mL, 392.80 ± 3.97 µg/mL, 187.15 ± 2.10 µg/mL, 361.43 ± 2..67 µg/mL, µg/mL and 393.2.2 ± 1.98 µg/mL respectively. However, PE fraction did not show DPPH scavenging activity.

MCE and DCM fractions revealed a significant ($P<0.01$) inhibitory action on α-glucosidase activity at concentrations ranging from 100 - 500 µg/mL. MCE and DCM

fractions exhibited α-glucosidase inhibitory activity at the IC_{50} of 397.49 ± 1.32 µg/mL and 264.94 ± 1.75 µg/mL respectively. However, PE, RM and sub fractions of DCM fraction such as fraction 1, fraction 2.1, fraction 2.2.1, fraction 2.2.2 & fraction 3 failed to inhibit α-glucosidase activity.

Methanol extract of *N. alata* and its fractions showed antiproliferative effect against human breast cancer cell line MDA-MB-231 and pancreatic cancer cell line PANC-1 in a dose dependant manner (Table 1).

Table 1. Effect of MCE and DCM fractions of *N. alata* on cancer cell lines MDA-MB-231 and PANC-1

Sample	Conc. (µg/mL)	% Inhibition	
		MDA-MB-231	PANC-1
MCE	200	No inhibition	25.72 ± 7.16
	100		No inhibition
	50		
	25		
	12.5		
DCM	200	64.63±2.16	58.58±1.08
	100	42.49±2.35	27.23±5.71
	50	27.21±4.63	16.09±1.54
	25	25.44±1.19	12.02±0.56
	12.5	19.04±1.61	6.08±1.22

*Values are the Mean ± standard deviation (SD).

Anti-inflammatory activities of the samples by HRBC membrane protection against hyposaline induction was in the following order: Fraction 2.2.1 with an EC_{50} value of 189.6 ± 2.61 µg/mL > DCM fraction with an EC_{50} value of 289.8 ±2.18 µg/mL > MCE with an EC_{50} value of 418.2 ± 2.71 µg/mL > fraction 1 with an EC_{50} value of 426.28 ± 3.17 µg/mL > fraction 2.1 with an EC_{50} value of 803.16 ± 4.89 µg/mL. The PE, RM and fraction 2.2.2 did not exhibit the protection of HRBC membrane.

The *N. alata* extract/fractions failed to show the *in vitro* glucose uptake by rat hemidiaphragm, inhibition of α- amylase activity and antimicrobial activity against any of the gram positive and gram negative bacteria tested.

4. Evaluation of *in vivo* antidiabetic activity of methanol extract, DCM fraction and its sub fractions of *N. alata*

4.1 Methodology

Induction of diabetes in rats and evaluation of antidiabetic activity

Three months old male Wistar albino rats weighing between 150 to 200 g, were acclimatised for one week before starting the experiment. They were maintained at standard laboratory conditions by placing 6 rats per cage and fed with rodent pellets and water *ad*

libitum. The rats were fasted for 8-12 h prior to the experiment, allowing access to water throughout the study period. The study protocols were evaluated and approved by the Institutional Animal Ethical Committee (MU/AZ/IAEC 2015-2016), Mangalore University, Mangalore, Karnataka, India.

Experimental rats were divided into following groups with six rats in each group.

Group I: Normal control + 1% Tween 20

Group II: Diabetic control + 1% Tween 20

Group III: Diabetic + MCE (1000 mg/ kg body weight)

Group IV: Diabetic + MCE (500 mg/ kg body weight)

Group V: Diabetic + DCM (100 mg/kg body weight)

Group VI: Diabetic + DCM (50 mg/kg body weight)

Group VII: Diabetic + Fraction 1 (40 mg/kg body weight)

Group VIII: Diabetic + Fraction 2.1 (40 mg/kg body weight)

Group IX: Diabetic + Fraction 2.2.1 (40 mg/kg body weight)

Group X: Diabetic + Fraction 2.2.2 (40 mg/kg body weight)

Group XI: Diabetic + Glibenclamide (20 mg/kg body weight)

Diabetes was induced by a single intraperitoneal injection of 60 mg/kg body weight of streptozotocin (STZ) dissolved in freshly prepared citrate buffer (0.1 M/L, pH 4.5) (Akbarzadeh et al., 2007). To evaluate the hypoglycaemic activity, the rats were administered with extract/fraction/standard drug as mentioned above for 21 days. The fasting blood samples were collected from tail vein at regular intervals of one week and glucose level was determined by Accu check Active (Model No: GB11404588, Germany). Body weight was measured using a digital weighing scale. The serum lipid parameters in the rat serum such as cholesterol (Allain et al., 1974), HDL-cholesterol (Gordon et al., 1977) and triglycerides (Jacobs, 1960) were estimated by using standard commercial kits purchased from Agappe Diagnostics Ltd., India. The concentrations of low density lipoprotein (LDL)-cholesterol and very low density lipoprotein (VLDL)-cholesterol were calculated from the formula (Friedwald et al., 1972).

The serum biochemical parameters such as urea was determined by modified diacetyl monoxime (DAM) method (Rahmatullah and Boyde, 1980), creatinine by Jaffe's reaction method (Toora and Rajagopal, 2002) and serum protein by Lowry et al. (1951) using bovine serum albumin (BSA) as standard. The liver antioxidant enzymes such as alkaline phosphatase was estimated by Otto et al. (1946) using the kit from Agappe diagnostics Ltd., India. The activity of serum glutamate oxaloacetate transaminase (SGOT)/Aspartate

transaminase (AST) and serum glutamate pyruvate transaminase (SGPT)/Alanine transaminase (ALT) were determined according to the method of Reitman and Frankel (1957). The catalase activity was determined by following Sinha (1972). The marker for free radical generation was determined in the serum using malondialdehyde estimation (Nadiger et al., 1986).

An oral glucose tolerance test was carried out in overnight fasted rats after 21 days of treatment with fractions of *N. alata* extract/fraction (Ng et al., 2015). The estimation of C-peptide in the serum of experimental rats were performed using Enzyme Immunoassay (EIA) kit based on the principle of competitive enzyme immunoassay. Liver glycogen content and glycogen synthase activity was determined following Hassid and Abraham (1957) and Leloir and Goldenberg (1979) respectively.

4.2 Results

Evaluation of hypoglycaemic activity

The effect of methanol extract/fractions of *N. alata* showed a significant ($P<0.01$) decrease in the blood glucose concentration in experimental rats as shown in Table 2. The DCM (100 mg/kg body weight), fraction 1 (40 mg/kg body weight) and fraction 2.2.1 (40 mg/kg body weight) was able to maintain the fasting blood glucose concentration of diabetic rats on par with normal control rats and better than standard drug treated diabetic rats throughout the experimental condition. There was a significant ($P<0.01$) improvement in the blood glucose concentration of diabetic rats treated with 1000 mg/kg of MCE and 50 mg/kg of DCM fraction compared to diabetic control groups.

The effect of methanol extract/fractions of *N. alata* showed a significant ($P<0.01$) improvement in the body weight of diabetic rats. The rats treated with 100 mg/kg of DCM, 40 mg/kg of fraction 1 and fraction 2.2.1 exhibited a significant ($P<0.01$) improvement in the body weight compared to diabetic control rats which was on par with diabetic rats treated with standard drug glibenclamide.

Hypolipidaemic activity

In the present study, MCE, DCM, fraction 1 and fraction 2.2.1 of *N. alata* showed hypolipidaemic activity in STZ induced diabetic rats. Diabetic rats treated with MCE (1000 mg/kg body weight and 500 mg/kg body weight) exhibited a significant ($P<0.01$) decrease in the TG, TC, LDL-cholesterol and VLDL-cholesterol concentrations compared to diabetic control rats. Diabetic rats treated with DCM at 100 mg/kg body weight, fraction 1 and fraction 2.2.1 at 40 mg/kg body weight showed a significant ($P<0.01$) decrease in the TG, TC, LDL -

cholesterol, VLDL-cholesterol concentrations compared to diabetic control rats and was better than diabetic rats treated with standard drug glibenclamide.

Table 2. Effect of *N. alata* extract/fraction on blood glucose concentration in experimental rats.

Groups	Blood glucose concentration of male Wistar rats on different days - mg/dL*				
	0	6	12	18	21
I	78.50±5.06^{a}	76.50±5.44^{a}	69.25±3.09^{a}	72.50±5.97^{a}	77.0±3.52^{a}
II	217.75±15.7^{b}	255.25±5.43^{d}	308.75±16.8^{c}	341±8.60^{d}	367.66±58.22^{d}
III	226±12.65^{b}	162±18.07^{c}	115.50±9.61^{b}	112±9.09^{b}	109.66±22.22^{b}
IV	223.25±10.7^{b}	179.25±13.7^{c}	124±6.73^{b}	138±22.37^{c}	136.33±17.08^{c}
V	230±14.33^{b}	96.75±15.86^{a}	83.75±11.76 ^{a}	91±5.59^{ab}	84.40±11.37^{a}
VI	231.25±12.0^{b}	121.25±4.99^{b}	104.25±12.6^{b}	112.75±3.6^{b}	94.50±14.84^{b}
VII	246±7.8^{b}	120.75±4.1^{b}	107±4.9^{ab}	103±5.12^{ab}	99.5±3.1^{b}
VIII	242±7.7^{b}	315.75±11.3^{e}	340.25±11.7^{d}	361±10.73^{d}	371.75±5.5^{d}
IX	234.25±4.9^{b}	232.5±10.6^{d}	143.5±6.4^{b}	112±6.28^{b}	96.5±4.7^{b}
X	237±9.9^{b}	308.75±9.1^{e}	336.75±8.6^{d}	351±9.81^{d}	376.5±7.9^{d}
XI	239.75±7.18^{b}	119±11.48^{b}	124.50±3.10^{b}	106±9.90^{b}	112.83±12.67^{b}

*Values are the Mean ± standard deviation (SD)

Furthermore, there was a significant (P<0.01) improvement in the HDL-cholesterol of diabetic rats treated with 1000 mg/kg of MCE, 500 mg/kg of MCE and DCM of 50 mg/kg body weight compared to diabetic control groups which were on par with HDL-cholesterol of diabetic rats treated with 20 mg/kg body weight of glibenclamide. Diabetic rats treated with DCM at 100 mg/kg body weight, fraction 1 and fraction 2.2.1 at 40 mg/kg body showed a significant (P<0.01) increase in the HDL cholesterol and the reversal was on par with that of control rats and better than standard drug glibenclamide treated diabetic rats (Fig 2).

Determination of serum protein, creatinine and urea

The results showed a significant (P<0.01) increase in the serum protein and decrease in the creatinine and urea concentrations of diabetic rats treated with MCE, DCM fraction at different concentrations and fraction 1 and fraction 2.2.1 at the concentration of 40 mg/kg body weight and the results were on par with that of diabetic rats treated with glibenclamide.

The results showed a significant (P<0.01) improvement in the liver and kidney protein concentrations of diabetic rats treated with MCE, DCM fraction at different concentrations

and fraction 1 and 2.2.1 at the concentration of 40 mg/kg body weight and the results were on par with that of diabetic rats treated with glibenclamide.

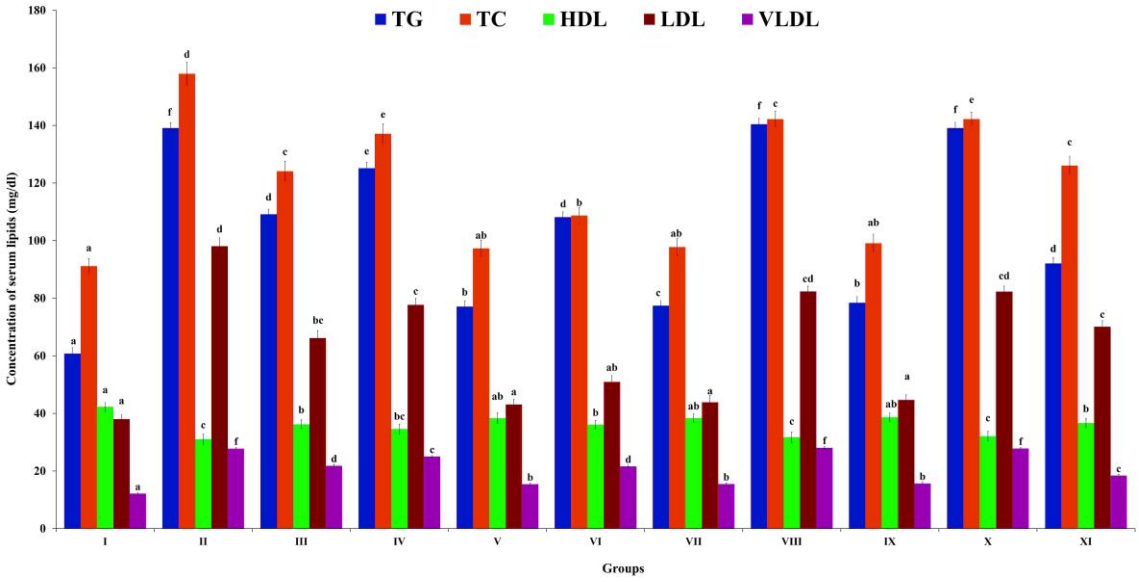

Fig 2. Effect of *N. alata* on serum lipid parameters in experimental rats.

In vivo antioxidant enzyme activity measured in the serum of experimental rats treated with *N. alata* extract/fraction showed a significant (P<0.01) reversal of ALP (alkaline phosphatase), AST (aspartate transaminase), ALT (alanine transaminase) and catalase compared to diabetic control rats. There was also a significant decrease in the malondialdehyde content in the diabetic rats treated with *N. alata* extract/fraction.

Oral glucose tolerance test

The oral administration of 100 mg/kg DCM fraction for 21 days to diabetic rats showed a significant (P<0.01) improvement in the oral glucose tolerance which was on par with the normal control groups after 3 hours of glucose administration. The DCM fraction (100 mg/kg body weight) decreased the blood glucose concentration in diabetic rats near to normal control (234.33 ± 4.72 mg/dL in 30 min to 106.66 ± 3.21 mg/dL in 3 hours) better than the standard drug throughout the experimental period. Fraction 1 and fraction 2.2.1 at the dose of 40 mg/kg body weight also showed a significant improvement in the oral glucose tolerance (233.33 ± 6.43 mg/dL in 30 min to 109 ± 2.3 mg/dL in 3 hours and 117.66 ± 2.51 mg/dL in 30 min to 107.66 ± 2.08 mg/dL in 3 hours respectively). Furthermore, there was a significant (P<0.01) improvement in the oral glucose tolerance of diabetic rats treated with 50 mg/kg of DCM fraction compared to diabetic control groups, which were on par with blood glucose of diabetic rats treated with 20 mg/kg body weight of glibenclamide (126.33 ± 3.05 mg/dL in 30 min to 136.66 ± 2.51 mg/dL in 3 hours).

Histopathological studies

Histopathology of pancreas of experimental rats carried out in the present study showed numerous pancreatic β-islets evenly distributed throughout and surrounded by the acinar cells in normal control rats. The diabetic rats showed damaged and shrunken islets with reduced size. The diabetic rats treated with MCE, DCM, fraction 1, fraction 2.2.1 and glibenclamide showed regeneration of islets (Fig 3). Histopathology of liver of experimental rats revealed a normal hepatic architecture with numerous closely packed hepatic cells around the narrow central vein and narrow sinusoids in normal control rats compared to the STZ induced diabetic rats. In STZ induced diabetic rats, inflammation around the central vein, dilation of sinusoid and central vein were observed along with the necrotic hepatic cells. Diabetic rats treated with both MCE and DCM fraction, fraction 1 and 2.2.1 showed an improvement in the architecture of liver by reduced dilation of sinusoidal and central vein and lesser necrotic liver cells. Similar results were observed in the case of diabetic rats treated with glibenclamide. Furthermore, DCM at 100 mg/kg body weight, fraction 1 and 2.2.1 at 40 mg/kg body weight treated diabetic rats showed comparatively better histological details with improved architecture of liver to other extract/fraction treated diabetic rats.

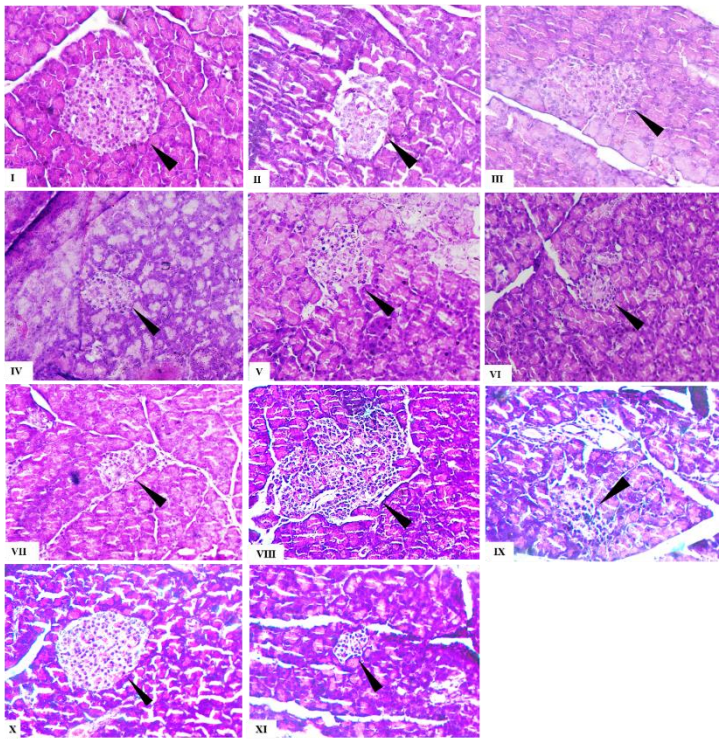

Fig 3. Effect of *N. alata* extract/fraction on histopathology of pancreas (scale 200 µm).

Histopathological studies carried out for kidney in experimental rats showed normal kidney architecture with glomeruli and convoluted tubules in normal control rats. Diabetic rats showed degenerated glomeruli and accumulation of fat in the medullary region. Whereas

diabetic rats treated with MCE (1000 mg/kg body weight), DCM (100 mg/kg body weight), fraction 1 (40 mg/kg body weight), fraction 2.2.1 (40 mg/kg body weight) and glibenclamide (20 mg/kg body weight) showed regeneration of glomeruli without fat accumulation.

Determination of serum C-peptide concentration, liver glycogen content and glycogen synthase enzyme

Diabetic rats revealed a significant (P<0.01) decline in serum C-peptide while a significant (P<0.01) increase in the C- peptide level was observed in the diabetic rats treated with DCM fraction (100 mg/kg body weight), fraction 1 (40 mg/kg body weight) and fraction 2.2.1 (40 mg/kg body weight) compared to the control rats. The improvement in the serum C-peptide levels was better than diabetic rats treated with standard drug glibenclamide (Fig 4).

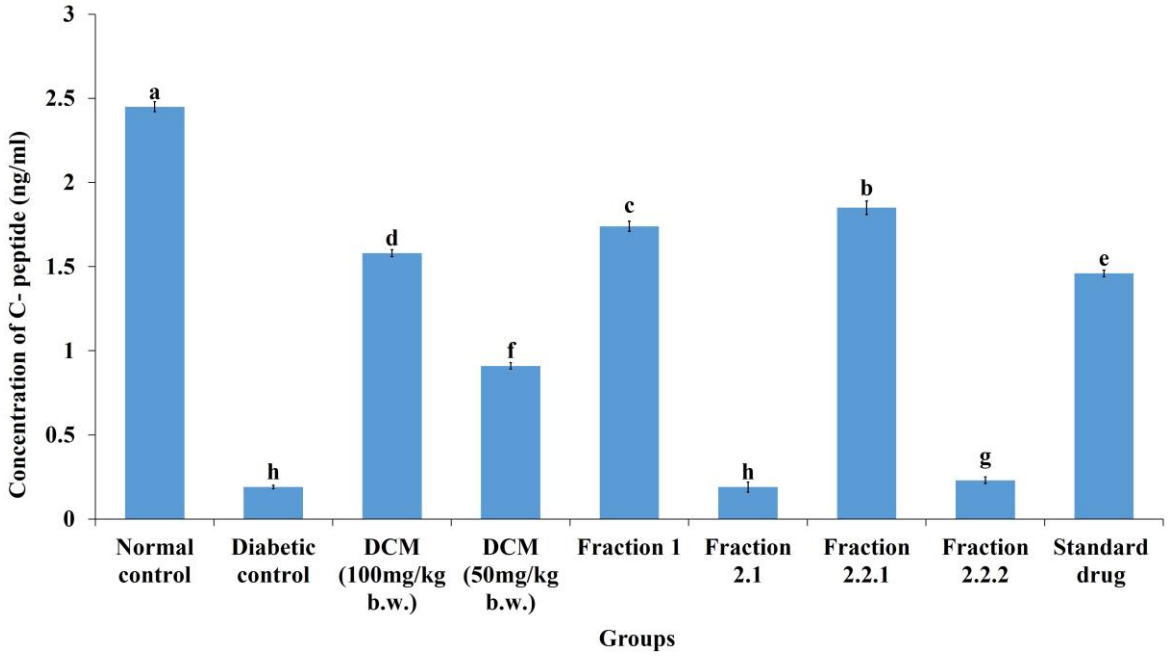

Fig 4. Effect of DCM fraction and its sub fraction on serum C-peptide levels in experimental rats.

Significantly (P<0.01) higher liver glycogen content was observed in the diabetic rats treated with DCM 100 mg/kg body weight, fraction 1 and fraction 2.2.1 at 40 mg/kg body weight compared to standard drug glibenclamide. However the diabetic rats treated with MCE (1000 mg/kg body weight) and DCM (50 mg/kg body weight) showed on par results with the glibenclamide treated groups. The diabetic rats treated with 500 mg/kg body weight also showed significant improvement in the liver glycogen content when compared to diabetic control rats.

There was a significant (P<0.01) increase in the glycogen synthase in diabetic rats treated with DCM at 100 mg/kg body weight and fraction 2.2.1 at 40 mg/kg body weight compared to diabetic control groups and the improvement was better than diabetic rats treated

with glibenclamide. Also, diabetic rats treated with fraction 1 at 40 mg/kg body weight revealed a significant increase in the activity of glycogen synthase compared to diabetic control groups and the improvement was on par with diabetic rats treated with glibenclamide. Diabetic rats treated with 1000 mg/kg of MCE and 50 mg/kg of DCM fraction also showed improvement in the glycogen synthase enzyme compared to diabetic control rats.

5. Chapter 3: Preliminary screening and characterisation of phytochemicals in *N. alata*

5.1 Methodology

Preliminary phytochemical analysis was carried out for *N. alata* extract/fraction to detect the presence of alkaloids, phenols, flavonoids, tannins, saponins, steroids, terpenoids and glycosides using standard methods (Harborne, 1998; Syed et al., 2012).

The quantitative estimation of phytochemicals such as phenolics (Malik and Singh, 1980), flavonoids (Durai et al., 2007), alkaloids (Sreevidya and Mehrotra, 2003) and terpenoids (Ghorai et al., 2012) in the *N. alata* extract/fraction was carried out.

Spectral analysis of fraction 1 and 2.2.1

Fraction 1 and 2.2.1, were subjected to spectral analysis to identify the phytochemicals present in the extracts. Fraction 1 and fraction 2.2.1 were subjected to GC-MS analysis at mass range of 10 - 2000 amu. The spectra of unknown components were compared with spectra stored in the National Institute Standard and Technology (NIST) library version 2. The eluted compounds were characterized based on their retention time and peak area in percentage. Fourier transform-infrared spectroscopy (FT-IR) analysis was performed (Shimadzu IR Prestige 21, Japan) to detect the functional groups in the range of 3000 - 400/cm. ^{1}H NMR spectra in DMSO for fraction 1 & 2.2.1 was measured.

5.2 Results

The phytochemical investigation carried out using methanol extract of *N. alata* and its various fractions showed the presence of different chemical constituents (Table 3). The quantitative analysis of phytochemicals revealed the highest amount of terpenoids in fraction 2.2.1 (703.71 ± 2.51 mg/g) and followed by the DCM fraction (695.94 ± 1.92 mg/g). The terpenoids present in fraction 1 was (198.1 ± 2.01 mg/g), fraction 2.1 (369.32 ± 2.32 mg/g), fraction 2.2.2 (441.3 ± 2.98 mg/g) and fraction 3 (295.35 ± 1.98 mg/g), MCE (202.49 ± 1.43 mg/g), PE (68 ± 1.2 mg/g) and RM (32 ± 0.87 mg/g). The highest amount of phenolics 81.16 ± 0.69 mg/g were recorded in fraction 2.2.1. followed by 71.07 ± 0.93 mg/g in fraction 1.

Table 3. Phytochemical screening of crude methanol extract and fractions of *N. alata*.

Phytochemicals	MCE	PE	DCM	RM	1	2.1	2.2.1	2.2.2	3
Alkaloids	+	-	+	+	-	-	-	-	-
Phenolics	+	-	+	+	-	-	+	-	+
Flavonoids	+	-	+	+	-	-	-	-	+
Saponins	+	-	+	+	-	-	-	-	-
Terpenoids	+	+	+	-	+	+	+	+	+
Steroids	+	+	-	-	+	+	-	+	+
Glycosides	+	-	-	+	-	-	-	-	-
Tannins	+	-	-	-	-	-	-	-	-

The amount of phenolics in the other samples were found to be 68.88 ± 0.70 mg/g in DCM fraction, 65.01 ± 0.60 mg/g in MCE, 50.45 ± 0.66 mg/g in fraction 2.2.2, (50.15 ± 0.95 mg/g in RM), and fraction 2.1 (41.87 ± 0.87 mg/g), fraction 3 (30.9 ± 0.73 mg/g) and PE (4.98 ± 0.58 mg/g). The highest amount of flavonoids 38.85 ± 0.72 mg/g was recorded in the DCM followed by MCE (32.86 ± 0.64 mg/g). The flavonoid content in the other samples were, RM (11.26 ± 0.59 mg/g), fraction 2.2.1 (41.17 ± 0.39 mg/g), fraction 2.2.2 (36.29 ± 0.39 mg/g) and fraction 3 (29.85 ± 0.41 mg/g), fraction 1 (19.03 ± 0.45 mg/g), fraction 2.1 (15.74 ± 0.32 mg/g) and PE (2.67 ± 0.57 mg/g). Furthermore, the highest amount of alkaloids were detected in the RM (52.16 ± 1.08 mg/g) and the second highest of alkaloids were detected in the MCE (34.99 ± 0.57 mg/g). A very low amount of alkaloids were detected in DCM (1.13 ± 0.11 mg/g) and PE (0.9 ± 0.02 mg/g).

The GC-MS analysis of fraction 1 detected the presence of a total of 25 compounds, of which 13-docosenamide was found to be a major compound with peak area, retention time and molecular mass of 68.27%, 27.046 and 337 respectively. The other major compound detected was hexadecanoic acid methyl ester with peak area, retention time and molecular mass of 12.77%, 17.959 and 270 respectively. The GC-MS analysis of fraction 2.2.1 detected the presence of a total of seven compounds of which, citronellol epoxide was the major compound with peak area, retention time and molecular mass of 29.92, 68.22 and 172 respectively.

FTIR spectrum of fraction 1 showed the presence of absorption bands for carbonyl stretching for esters (2999-2800), CH_2-CH_2 stretching (2840-2860) and methyl bending (1477-1175). The FTIR spectrum of fraction 2.2.1 showed the presence of absorption bands for polymeric OH stretch (3600–3200), methylene C-H asymmetric stretch (2935–2915),

unsaturation (1670-1620), methylene C-H bend (1485-1445), methylene C-H symmetric bend (1380-1371), C-O stretch (1140-1050), 1, 3 disubstitution (860-800).

The ^{1}H NMR carried out for fraction 1 revealed that the chemical shifts were observed at 3.626 (s, 3H), 2.264 (m, 2H), 1.496 (2H), 1.233 (m, 2H), 0.851 (t, 3H) which corresponds to the hexadecanoic acid methyl ester and chemical shifts were observed at 0.834 (d, 3H), 1.496 (m, 2H), 0.996 (m, 2H), 5.319 (d, 1H) corresponds to the 13- docosenamide and ^{1}H NMR carried out for fraction 2.2.1 revealed that the chemical shifts were observed at 3.296 (d, 2H), 1.466 (t, 2H), 1.157 (t, 1H), 1.368 (d, 2H), 2.167 (t, 2H), 5.312 (m, 1H), 1.225 (s, 3H) corresponds to the citronellol.

6. Chapter 4: *In silico* evaluation of pharmacokinetics, drug-likeness and medicinal chemistry of citronellol, hexadecanoic acid methyl ester and 13-docosenamide

6.1 Methodology

The pharmacokinetics, drug-likeness and medicinal chemistry of citronellol, hexadecanoic acid methyl ester, and 13-docosenamide were predicted by using the SwissADME website (http://swissadme.ch) (Ibrahim et al., 2021; Mvondo et al., 2021).

6.2 Results

Citronellol and hexadecanoic acid methyl ester were predicted to have a high GI (Gastrointestinal absorption) but to cross the blood-brain barriers, while 13-docosenamide is predicted to have low GI absorption and not to cross the blood brain barrier. All the three isolated compounds were predicted as not substrates for P-gp. The molecules did not show any inhibition of the tested CYP isoforms excepting hexadecanoic acid methyl ester and 13-docosenamide on CYP1A2 as per the prediction results. The predicted Ilogp values of citronellol, hexadecanoic acid methyl ester, and 13-docosenamide are 2.72, 4.41, and 5.10 respectively. Citronellol agrees Lipinski's rule of five, but hexadecanoic acid methyl ester and 13-docosenamide were predicted to violate Lipinski's rule of five due to the MLOGP (Moriguchi octanol-water partition coefficient) > 4.15. All the three compounds analysed failed to pass Ghose's rule, due to MW (Molecular weight)<160 for citronellol, WLOGP (Wildman and Crippen water partition coefficient)>5.6 for hexadecanoic acid methyl ester, and 13-docosenamide. The citronellol and hexadecanoic acid methyl ester compounds pass Egan's rule but 13 -docosenamide violates Egan's rule due to WLOGP>5.88. The citronellol predicted to clear the Veber rule. The hexadecanoic acid methyl ester and 13 - docosenamide violate the Veber rule due to the number of rotatable bonds being more than 10. None of the molecules analysed agree with Muegge's rule due to the MW<200, Heteroatoms<2 in citronellol, XLOGP3>5 in hexadecanoic acid methyl ester, and XLOGP3>5, Rotors>15 in 13-

docosenamide. All the three molecules are predicted to have a topological polar surface area (TPSA) of ≤ 140 $Å^2$. All the three molecules analysed are predicted to clear the PAINS alert. Hexadecanoic acid methyl ester clears the Brenk alert but citronellol and 13-docosenmide violate the Brenk alert by the formation of isolated alkenes. All the screened compounds predicted a relatively good bioavailability score of 0.55 when administered orally. All the three molecules were predicted to violate the Leadlikeness. Among the three compounds, citronellol was found within the data range as predicted with pink colour in the SwissADME bioavailability radar. But Hexadecanoic acid methyl ester and 13-docosenamide show higher flexibility and lipophilicity deviating from the radar zone as indicated in the SwissADME bioavailability radar.

7. Discussion

In the present study, a significant (P<0.01) *in vitro* antioxidant activity was observed in MCE and its fractions obtained from *N. alata* and these results are in agreement with the earlier reports on the antioxidant activity of *N. alata* plant extract conducted by Joseph et al. (2010) and Velayudhan et al. (2015). The present study showed a significant (P<0.01) inhibitory action on α- glucosidase activity by MCE and DCM fraction. The inhibition of α - glucosidase activity will result in decrease in the absorption of glucose, leads reduction of postprandial blood glucose level (Banerjee et al., 2017). *N. alata* extract/fraction showed the stabilisation of HRBC membrane from hypotonic lysis indicating that these fractions may act as an anti-inflammatory. The present findings suggest that the methanol extract and its DCM fraction might act as promising antitumor agents against pancreatic and breast cancer. Earlier studies proved that extracts with antioxidant activities also exhibit anti-inflammatory and anticancer activities (Altundag et al., 2020).

The reversal of blood glucose concentrations in STZ induced diabetic rats treated with *N. alata* extract/fraction in the present study might be due to the presence of bioactive compounds. Similar results were obtained in earlier studies such as methanol extract of *Piper Claussenianum* flowers (Marques et al., 2015), methanol extract of *Syzygium polyanthum* (Wight.) leaf (Widyawati et al., 2015) etc. The significant improvement in the body weight of diabetic rats indicting the utilization of glucose as a main source of energy hence preventing the loss of tissue proteins (Rajasekar et al., 2014). Treatment of diabetic rats with *N. alata extract/fractions* reversed dyslipidemia as evidenced by the significant decrease (p<0.01) in triglycerides, total cholesterol, LDL-cholesterol, VLDL-cholesterol and increase in HDL-cholesterol denotes the antihyperlipidaemic potential of *N. alata.* Many plant extracts have been reported for their antidiabetic activity with reversal of dyslipidemia such as *Uvaria*

chamae root (Schofield et al., 2016), Fruits and roots of *Curculigo latifolia* (Ishak et al., 2013) etc.

The increase in the levels of serum creatinine and urea during diabetes mellitus causes renal failure known as diabetic nephropathy (Singh et al., 2014; Chutani and Pande, 2017). The current results with *N. alata* extract/fraction showed an improved renal dysfunction in STZ induced diabetic rats through a decrease in the serum urea and creatinine level which in turn decreases the risk of diabetic nephropathy. Chronic diabetes mellitus exhibits an elevation of activity of AST, ALT, ALP and decrease in catalase activity (Mathur et al., 2016). The current study with the administration of *N. alata* extract/fraction showed an increase in the serum catalase activity and decrease in the activity of serum AST, ALT, and ALP in diabetic rats indicating its antioxidant property against the reactive oxygen species produced during chronic hyperglycaemia. There was an increase in the level of malondialdehyde in STZ induced diabetic rats and a decrease in the malondialdehyde content in *N. alata* extract/fraction treated rats further proving the antioxidant property of this plant and also protection against cardiovascular risk factors caused by diabetes mellitus (Tangvarasittichai et al., 2009).

The histopathological study showed an increase in the volume density of islets, percent of β cells, and size of the islet in the groups that received the plant extract/fraction indicating regeneration of the pancreas with β-cells repairs. Similar results were reported in plant extracts of *Spondias pinnata, Coccinia grandis, Gmelina arborea* (Attanayake et al., 2018), and *Gymnema sylvestre* leaf extracts (Shanmugasundaram et al., 1990). The increase in the serum C-peptide in diabetic rats further suggest that active fraction might have triggered the secretion of insulin from the existing β-cells of the pancreas and probably the plant fractions also helped in the regeneration of the β-cells of the pancreas destructed by STZ (Fouhil, et al., 2013). Glycogen synthase is an enzyme, activated by insulin and converts glucose into glycogen, resulting in the regulation of blood glucose levels (Oyenihi et al., 2019). The ability of *N. alata* to ameliorate glycogen levels indicates that this plant has the potential to regenerate the pancreatic *β*-cells and increase the secretion of insulin levels, hence enhancing the storage of glucose as glycogen via glycogenesis. The improvement in the architecture of the liver of diabetic rats might be due to the antioxidant property of *N. alata* to reduce the damage caused by STZ as reported earlier in the plant extracts of *Allium sativum* L. (Eidi et al., 2006), and *Tectona grandis* (Ramachandran et al., 2011). Diabetic rats treated with *N. alata* extract/fractions further confirmed the protective effect of bioactive molecules

of *N. alata* against diabetic nephropathy through the regeneration of glomeruli without fat accumulation (Madhan et al., 2016).

The current study agrees with the previous study conducted by Hidayathulla (2012), wherein a wide range of phytochemicals were extracted by using the soxhlet method and methanol as solvent. The presence of different phytochemicals in *N. alata* might be responsible for their *in vitro* pharmacological activity such as antiinflammatory, antioxidant, cytotoxic, and *in vivo* antidiabetic activity. Further characterisation of the active fractions proved that the presence of 13-docosenamide and hexadecanoic acid methyl ester are the major compounds in fraction 1 and citronellol in fraction 2.2.1. 13-docosenamide has been previously detected as the major phytoconstituent present in the ethanolic seed extract of *Annona reticulata* L. with antidiabetic activity in STZ induced type 2 diabetic animal model (Wen et al., 2019). The earlier studies conducted on citronellol using STZ induced diabetic animal model proved its antidiabetic activity with significant improvement in the blood glucose concentration, insulin level, haemoglobin, glycated haemoglobin and liver glycogen in diabetic rats (Srinivasan and Muruganathan, 2016). Hence the presence of citronellol in fraction 2.2.1 might be responsible for its antidiabetic activity.

The swissADME druglikeness property of the isolated molecules from *N. alata* depicts that, citronellol can be a good candidate for the development of drugs by obeying the Lipinski's rule of five and Veber's rule, Egan's rule and also citronellol falls within the radar zone of the bioavailability predicting to be orally active (Daina and Zoete, 2019.). The current study proves that *N. alata* is a potent antidiabetic herb and the compounds isolated from this plant can be therapeutic antidiabetic agents. However, further detailed evaluation is required for accurate hypoglycaemic activity of these compounds to treat various types of diabetic mellitus. These results support for the clinical studies to be taken up using the extracts/fraction of *N. alata* which miht prove helpful in the management of diabetes mellitus.

Research outcomes

- *In vitro* antioxidant activity of *N. alata* extract/fractions.
- *In vivo* antidiabetic activity of extract/fractions of *N. alata* carried out using STZ induced diabetic rats revealed a significant ($p<0.01$) improvement in blood glucose level and body weight.
- Reversal of dyslipidaemia by *N. alata* extract/fraction.

- Improvement in the biochemical parameters such as urea and creatinine by *N. alata* extract/fraction.
- Regeneration of pancreas, improvement in the architecture of liver and kidney due to the treatment of *N. alata* extract in STZ induced diabetic rats.
- Insulinotropic and glyconeogenesis property of *N. alata*.
- Presence of polyphenols, alkaloids, saponins, flavonoids, glycosides, terpenes, steroids and tannins in the extracts of *N. alata*.
- The presence of citronellol, 13-docosenamide and hexadecanoic acid methyl ester isolated from *N. alata* might be responsible for its antidiabetic property.

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9. List of publications

- Wilson Joel Rodrigues, Bhagya N, Chandrashekar K R. Antidiabetic activity of *Naregamia alata* in streptozotocin induced diabetic rats and associated mechanism of action. Synergy, 11, p.100064.
- Wilson Joel Rodrigues, Bhagya N, Chandrashekar K R, Boja Poojary. Bioassay-guided isolation and identification of antidiabetic compounds from *Naregmaia alata*. Current bioactive compounds (Under review).
- Wilson Joel Rodrigues, Bhagya N, Chandrashekar K R. Insuilnotropic activity of antidiabetic compounds isolated from *Naregmaia alata* (under preparation).
- Wilson Joel Rodrigues, Bhagya N, Chandrashekar K R. Antioxidant property of *Naregmaia alata* extract and its active fractions in streptozotocin induced diabetic rats (under preparation).
- Wilson Joel Rodrigues, Bhagya N, Chandrashekar K R. *In silico* evaluation of pharmacokinetics, drug-likeness and medicinal chemistry of citronellol, hexadecanoic acid methyl ester and 13-docosenamide (under preparation).

10. List of papers presented in conferences

- Wilson Joel Rodrigues, Bhagya N, Chandrashekar K R. *In vivo* antidiabetic activity of *Naregamia alata.* National conference on biological on basic and applied biological sciences. Bharathiar University, Coimabatore, tamil Nadu, 10^{th} -11^{th} October 2018.
- Wilson Joel Rodrigues, Bhagya N, Chandrashekar K R. Effect of *Naregamia alata* on biochemical parameters of STZ induced diabetic rats. Emerging Perspectives in Preclinical and Clinical Research, NGSMIPS, Nitte (Demmed to be University), Mangalore, Karnataka, 28^{th} - 29^{th}March 2019.
- Wilson Joel Rodrigues, Bhagya N, Syed Hidayathulla, Chandrashekar K R. Antihyperlipidemic activity of *Naregamia alata* in STZ induced diabetic rats. 7th Annual Symposium Society of Biological chemists (India), Coastal Karnataka Chapter,

Manipal school of Life Sciences, Manipal Academy of Higher Education, 23rd November, 2019.

- Wilson Joel Rodrigues, Bhagya N, Chandrashekar K R. Evaluation of hepatic glycogen content in STZ induced diabetic rats treated with *Naregamia alata*. International conference on Synergy of Sciences, Sastra Deemed to be University, Thanjavoor, Tamilu Nadu, 27-29 Febraury 2020.

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